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Award Number: DAMD17-00-1-0339

TITLE: Development of Triplex-Forming Oligonucleotides to Inhibit Expression of the c-myc Oncogene in Breast Cancer Cells

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REPORT DATE: April 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2002	Annual Summary (6 Mar 01 - 5 Mar 02)

4. TITLE AND SUBTITLE
Development of Triplex-Forming Oligonucleotides to Inhibit Expression of the c-myc Oncogene in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-00-1-0339

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Triple helix-forming oligonucleotides (TFOs) directed to regulatory sequences in gene promoters can selectively block transcription. We are investigating TFO-mediated reduction of c-myc oncogene expression as a means of decreasing breast tumor growth. We designed a novel parallel/antiparallel TFO (Myc-GTC) that had high binding affinity *in vitro,* but required modifications for increased stability in cells. To further optimize TFO activity we investigated effects of conjugation with the anthracycline antibiotic daunomycin (Dnm), which intercalates into double-stranded DNA. We synthesized short Dnm-conjugated TFOs corresponding to parallel and antiparallel elements of Myc-GTC. Electrophoretic mobility shift and footprinting assays showed that Dnm-TFOs formed highly specific, stable triplex. Dnm-TFOs inhibited binding to the target duplex of transcription-activating proteins present in MCF-7 nuclear extracts. Fluorescence microscopy determined that Dnm-TFOs were efficiently internalized by MCF-7 cells. At nanomolar concentrations, a Dnm-TFO inhibited expression in MCF-7 and MDA-MB-231 cells of a luciferase gene under the control of the *c-myc* promoter. These results suggest that Dnm-conjugated TFOs are effective in cells, and that Dnm may stabilize binding of the full-length parallel/antiparallel TFO in cells.

14. SUBJECT TERMS
Breast cancer therapy, gene-specific experimental Oligonucleotides, triple helical DNA, c-Myc

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Description of training and research accomplishments.

The approved proposal described three tasks. Progress made on each task during the second year is described below, following a general introduction to the use of synthetic triple helix-forming oligonucleotides (TFOs) and a short overview of progress made in our investigation.

Introduction

Triple helix formation offers a direct means of selectively manipulating gene expression in cells. TFOs bind with high affinity and specificity to the purine strand in the major groove of homopurine-homopyrimidine sequences in double-stranded DNA. TFOs have proved effective in various gene-targeting strategies in living cells and, recently, in animals. TFOs targeted to regulatory sequences in target genes have been able to selectively reduce gene transcription. This approach has potential application in anti-cancer treatment since it can be used to reduce levels of proteins essential to proliferation of cancer cells.

C-Myc plays a central role in promoting cell growth and proliferation. The *c-myc* gene is expressed at abnormally high levels in many cancers, and its overexpression has been strongly associated with initiation and progression of breast cancer. Reduced levels of c-Myc are associated with decreased breast cancer cell growth. The purpose of our investigation is to assess triplex DNA-mediated reduction of *c-myc* expression as a potential means of decreasing growth and spread of breast tumors.

The research described in the approved proposal builds on previous studies conducted in the mentor's laboratory and elsewhere. In a study comparing TFOs directed to various polypurine sequences in *c-myc*, we found that a TFO targeted to a critical regulatory region close to the P2 promoter had strong anti-gene and anti-proliferative effects in leukemia and lymphoma cells. These findings encouraged further development of this TFO. The specific problems addressed by the present research arose from modifications required to confer nuclease resistance on the c-myc TFO. Nuclease resistance is required in TFOs intended for *in vivo* use. Replacement of nuclease-sensitive phosphodiester (PO) internucleotide linkages with resistant phosphorothioate (PS) appeared to reduce the binding affinity of the c-myc TFO. Furthermore, adverse effects of high concentrations of PS-oligonucleotides have been observed in cells and animals. We therefore decided to investigate modifications in design, which could enhance TFO binding affinity, and retain nuclease resistance while allowing reduced PS content.

Overview

As described in the 2001 annual report we synthesized a TFO, Myc GTC, with novel structural properties and significantly improved *in vitro* binding affinity compared to TFOs previously directed to the target site in the *c-myc* major promoter. The parallel/antiparallel design was clearly optimal for triple helix formation on the c-myc sequence. In the current year, gel shift assays with breast cancer cell nuclear extracts confirmed that MycGTC blocked binding of transcriptional activators Sp1 and Sp3 to the *c-myc* P2 sequence. We are currently evaluating modifications to ensure stability against nuclease degradation in cells, since we found that the double 5' ends provided protection against nucleases in serum, but did not prevent rapid degradation in breast

cancer cells. To further optimize TFO activity we investigated effects of conjugation with a DNA intercalating agent. Binding studies showed that TFOs conjugated to the anthracycline antibiotic daunomycin (Dnm) formed more stable triple helix than their unconjugated counterparts. In shift assays with nuclear extracts Dnm-TFOs inhibited binding of proteins to the target duplex more efficiently than unconjugated TFOs. Furthermore, Dnm-TFOs were efficiently internalized by MCF-7 cells, as determined by fluorescence microscopy, and at nanomolar concentrations, inhibited expression in breast cancer cells of a *c-myc* promoter-driven luciferase gene. Conjugation to Dnm is a promising novel strategy for stabilizing the triple helix in vitro and in cells. It is anticipated that technical advances will allow synthesis in 2002 of Dnm-conjugated Myc GTC.

Progress on tasks.

Task 1. To design and evaluate activity of c-myc TFOs optimized for nuclease resistance and high-affinity DNA binding.

TFOs can bind either parallel or antiparallel to the purine-rich strand of polypurine/polypyrimidine sequences in double-stranded DNA. The myc P2 target sequence is evenly divided into two regions, which we found to favor binding in opposite orientations. To optimize binding to the entire sequence, we synthesized a parallel/antiparallel TFO with a central 3'-3' interface. This TFO, Myc GTC, had 5-10 fold improved binding affinity in vitro compared to anti-parallel TFOs. The requirement for cytosine protonation for binding in the parallel motif was addressed by including methylated cytosines in the parallel moiety of the novel TFO. The TFO's double 5' ends conferred resistance to digestion by nucleases present in fetal bovine serum, but afforded only partial, short-term protection against nuclease degradation when the TFO was transfected into breast cancer cells.

As a preliminary test of TFO activity in the cellular environment, we extracted proteins from MCF-7 and MDA-MB-231 breast cancer cell nuclei and used gel shift assays to study protein binding to the target sequence with and without preformed triplex. Competition and antibody analysis identified transcriptional activators Sp1 and Sp3 forming the major gel-shifted bands. When the 40 bp target duplex was pre-incubated for 2 h with 1 μM Myc GTC, binding of Sp1 and Sp3 was reduced by over 50%. An antiparallel TFO inhibited binding by less than 20%, and control oligonucleotides had no effect. To confirm the specificity of this inhibitory activity, we showed that Myc-GTC did not prevent Sp1 and Sp3 binding to a sequence in the Myc P1 promoter.

We have also used gel shift assays to test activity of TFOs conjugated to daunomycin (Dnm). So far, we have synthesized short Dnm-TFOs corresponding to the parallel and antiparallel segments of Myc-GTC, as well as a full-length antiparallel TFO. In all experiments to date, binding stability and inhibitory activity of Dnm-TFOs was increased compared to non-conjugated counterparts, with no loss of specificity. Since the Dnm short TFOs form highly stable triplex on only half of the target sequence, they may be useful to identify proteins binding to different regions. We have so far confirmed that Sp1 and Sp3 binding is strongly inhibited by a TFO targeted only to the 11 bp G-rich region. We anticipate that technical advances will allow synthesis of Dnm-conjugated Myc-GTC in coming year.

Task 2. To enhance efficiency of cell internalization, maximize stability, and compare growth-inhibitory activities of PS- and modified TFOs in estrogen-dependent and – independent breast cancer cells.

We studied uptake and integrity of a short antiparallel Dnm-conjugated TFO in MCF-7 breast cancer cells. Dnm can be detected using fluorescence microscopy. We found that the TFO in the absence of a transfection reagent was taken up into cells with efficiency comparable to free Dnm. When a cationic lipid reagent was used, intracellular levels of Dnm-TFO increased dramatically. Free Dnm localized predominantly to cell nuclei, and was detectable as homogeneous fluorescence. The Dnm-TFO was concentrated in brightly fluorescent foci, which appeared to be mainly cytoplasmic. Transfected Dnm-TFO was detectable at similar levels for at least 24 h, whereas free DNM and TFO delivered without lipid were markedly depleted at 24 h. These results suggested that uptake and intracellular transport of Dnm-TFO was determined by the DNA, not the anthracycline moiety. It also seemed unlikely that any significant amount of DNM had become detached from the DNA, since it would presumably have been detectable in nuclei. Furthermore, observation of cells by phase contrast showed morphological changes at 24 h in Dnm-treated cells, probably reflecting loss of viability caused by treatment with free drug. Morphology of cells treated with Dnm-TFO was unaltered at this time point compared to 6 h, indicating that divergent trafficking of the two agents resulted in different intracellular activity. We conclude that Dnm is a suitable agent for enhancing TFO activity in cells, and that Dnm-Myc GTC will be an effective antigene agent.

Task 3. To extensively characterize anti-gene activity, growth inhibitory activity, and triplex forming ability of TFOs in breast cancer cells.

As previously reported, we found that a short Dnm-TFO inhibited expression in breast cancer cells of the luciferase gene driven by the *c-myc* P2 promoter. Similar experiments with Myc-GTC will be done when modifications for nuclease-resistance have been incorporated, and when synthesis of a Dnm-conjugated TFO has been accomplished.

Key accomplishments.

- TFOs conjugated to the anthracycline antibiotic daunomycin have been synthesized and extensively tested *in vitro* and in breast cancer cells
- Uptake and stability of daunomycin-conjugated TFOs in breast cancer cells has been demonstrated.
- Nuclear extracts from breast cancer cells have been prepared and used in assays to show specific inhibition of transcription factor binding by various TFOs.
- Further characterization of the parallel/antiparallel TFO, Myc-GTC has confirmed that this design is optimal for targeting the *c-myc* P2 promoter.

Reportable outcomes.

Papers.

McGuffie, E.M. and Catapano, C.V. 2002. Design of a novel triple helix-forming oligodeoxyribonucleotide directed to the major promoter of the *c-myc* gene. Nucleic Acids Research. Manuscript submitted.

Catapano, C.V., McGuffie, E.M., Carbone, G.M.R., Flanagan, C.E., Dembech, C., Arcamone, F., and Capobianco, M.L.. Synthesis of daunomycin-conjugated triplex-forming oligonucleotides that act as selective repressors of c-myc gene expression. 2002. Manuscript in preparation.

Abstracts

McGuffie, E.M. and Catapano, C.V.C. Novel design of an antigene triple helix-forming oligonucleotide directed to the *c-myc* major promoter. 2002. Proc. AACR. 43: 578